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Title: Transcriptome profiling reveals the crucial biological pathways involved in cold response in Moso bamboo (*Phyllostachys edulis)*

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Abstract

Most bamboo species including Moso bamboo (*Phyllostachys edulis*) are tropical or subtropical plants that greatly contribute to human wellbeing. Low temperature is one of the main environmental factors restricting bamboo growth and geographic distribution. Our knowledge of the molecular changes during bamboo adaption to cold stress remains limited. Here, we provided a general overview of the cold-responsive transcriptional profiles in Moso bamboo by systematically analyzing its transcriptomic response under cold stress. Our results showed that low temperature induced strong morphological and biochemical alternations in Moso bamboo. To examine the global gene expression changes in response to cold, 12 libraries (non-treated, cold-treated 0.5 h, 1 h and 24 h at -2°C) were sequenced using an Illumina sequencing platform. Only a few differentially expressed genes (DEGs) at early stage while a large number of DEGs at late stage were identified in this study, suggesting that the majority of cold response genes in bamboo are late-responsive genes. A total of 222 transcription factors from 24 different families were differentially expressed during 24h cold treatment, and the expressions of several well-known C-repeat/dehydration responsive element-binding factor (CBF) negative regulators were significantly up-regulated in response to cold, indicating the existence of special cold response networks. Our data also revealed that the expression of genes related to cell wall and the biosynthesis of fatty acids were altered in response to cold stress, indicating their potential roles in the acquisition of bamboo cold tolerance. In summary, our studies showed that both plant-kingdom conserved and species-specific cold response pathways exist in Moso bamboo, which lays the foundation for studying the regulatory mechanisms underlying bamboo cold stress response and provides useful gene resources for the construction of cold-tolerant bamboo through genetic engineering in the future.

Introduction

Bamboo is one of the most important non-timber forest products, covering over 30 million hectares (ha) worldwide and accounting for 68.8 billion US dollars in international trade in 2018 (King 2019). Moso bamboo (*Phyllostachys edulis*), is one of the most economically important bamboo species, serves as a promising bio-resource for renewable forestry products, and accounts for over two-thirds of total bamboo growing area (4.43 million ha) in China (Peng et al. 2013). The distribution of bamboo in nature is greatly influenced by agro-climatic zones, human interventions, and climatic factors (Lucina Yeasmin et al. 2017). Most bamboo species, including Moso bamboo, are commonly located in tropical or subtropical climatic regions, and temperature is one of the major environmental factors that control Moso bamboo growth and geographic distributions (Gu et al. 2010; Numata et al. 1957; Wenwei 1991; Xu and Qin 2003; Yeasmin et al. 2015). Over the past two decades, significant progress has been made in understanding the plant responses to chilling (0–15 °C) and freezing (<0 °C) in *Arabidopsis thaliana* (Lee et al. 2005), rice (Zhang et al. 2014), cotton (Kargiotidou et al. 2010), soybean (Calzadilla et al. 2016) and tomato (Weiss and Egea-Cortines 2009). In contrast, our understanding of the mechanisms that underlie bamboo cold stress response is surprisingly limited.

In the currently accepted model, cold stress first acts on the signal perception and transduction pathways, which induces transcriptional control, and consequently activates a variety of cold-regulated (COR) proteins (Guo et al. 2018; Zhu 2016). Molecular, physiological and metabolic studies demonstrated that low temperature leads to changes in membrane fluidity, initiating the cellular cold response through calcium (Ca^{2+}) signaling pathways (Sangwan et al. 2001; Zhang et al. 2014). Ca^{2+} is recognized by calcium-binding proteins such as calmodulin (CaM), CaM-like proteins (CML), Ca^{2+} dependent protein kinases (CDPKs), and calcineurin B-like proteins (CBLs) (Kudla et al. 2018). These calcium-binding proteins also known as Ca^{2+} sensors are induced early (3 h of exposure to 0 °C) during cold stress in Arabidopsis and rice (Abbasi et al. 2004; Lee et al. 2005). Mitogen-activated protein kinase

(MAPK) cascades, which are activated by various stress signal messengers, also play a role in cold response. A typical MAPK cascade is composed of three protein kinases: MAP kinase kinase kinase (MAPKKK or MEKK), MAP kinase kinase (MAPKK, MKK, or MEK), and MAP kinase (MAPK or MPK). Among them, MKK2 induces the expression of *COR* genes to enhance freezing tolerance in Arabidopsis (Teige et al. 2004). MPK3/6, on the other end, negatively regulates freezing tolerance via phosphorylation and destabilization of the inducer of *CBF* expression 1 (ICE1), which is a basic-helix-loop-helix (bHLH) transcription factor and acts as the master regulator of cold response in Arabidopsis (Li et al. 2017; Zhao et al. 2017). The integration of these signals is mediated through the coordination of transcriptional activators and repressors, many of which have been well characterized (Chinnusamy et al. 2007). The typical transcriptional regulation pathways of cold stress are C-repeat binding factor (CBF)-dependent and CBF-independent pathways. The key components of the CBF-dependent pathway are ICE1-CBF-COR, and they play a predominant role in cold tolerance (Guo et al. 2018; Zhou et al. 2011). ICE1 mediates the CBF-dependent pathway by positively regulating the expression of *CBFs* (Guo et al. 2018). Overexpression of *ICE1* leads to an increased expression of *CBFs* and improves cold tolerance in transgenic Arabidopsis (Chinnusamy et al. 2003). A large number of genes directly or indirectly participate in cold regulation through regulating ICE1 at the level of transcription, translation and post-translation (Agarwal et al. 2006; Maruyama et al. 2014). CBFs bind to the promoter of *COR* genes to activate their expression and confer increased freezing tolerance in plants (Gilmour et al. 2004). CBF homologs have been characterized in many plant species such as rice (Dubouzet et al. 2003), maize (Qin et al. 2004), barley (Morran et al. 2011) and soybean (Kidokoro et al. 2015). Transgenic plants overexpressing *CBFs* show enhanced cold tolerance compared to wild type (Ito et al. 2006; Kasuga et al. 2004). The expressions of *CBFs* are also negatively regulated by a number of transcription factors such as *MYB15* and *ZAT12* (Agarwal et al. 2006; Maruyama et al. 2009).

Plant adaption to cold stress involves changes at cellular and molecular levels, which are governed by plant hormones (Lado et al. 2016). Abscisic acid (ABA) is the

key plant hormone that is involved in plant responses to abiotic stresses (Gusta and Wisniewski 2013). In many species, cold stress is accompanied by the increased expression level of the 9-cis-epoxycarotenoid dioxygenase (*NCED*) gene, which encodes one of the key enzymes for ABA biosynthesis and leads to the induction of endogenous ABA (Mantyla et al. 1995). The ABA signal is perceived through the ABA receptor complex, which is composed of PYRABACTIN RESISTANCE 1 (PYR1), PYR1-like protein (PYL) and regulatory components of the ABA receptor (RCAR) family of START proteins, and induces broad gene expressions in response to abiotic stresses (Lee and Luan 2012). The current consensus is that both ABA-dependent and ABA-independent pathways are involved in the plant responses to cold stress (Lado et al. 2016). The ethylene pathway seems to play a negative role in regulating freezing tolerance partly by inhibiting the functions of *CBF* or *DREB* in Arabidopsis (Kazan 2015; Shi et al. 2012). Recent reports showed that the ethylene pathway also plays a positive role in cold stress in Arabidopsis, tomato, rice and tobacco (Catala and Salinas 2015; Tian et al. 2011; Zhang et al. 2009) . Therefore, the role of ethylene in cold tolerance appears to be species dependent. A key plant response to cold is growth repression, through which plants might re-allocate resources from growth to processes that help to increase cold tolerance (Eremina et al. 2016). Gibberellins (GAs) are well-known growth promoting hormones, and both GA metabolism and signaling are targeted by cold stress (Achard et al. 2008; Seo et al. 2009). Cold induces the expression of *GA 2-oxidases* (*GA2OX*) gene, which encodes a key enzyme for the inactivation of bioactive GAs (Xu et al. 1999). Studies from Arabidopsis revealed that CBF3 promotes the accumulation of DELLA proteins, which are key negative regulators in GA signaling pathway, and lead to retarded plant growth in response to cold (Zhou et al. 2017). Auxin not only plays vital roles in plant growth and development, but also mediates the cold response (Rahman 2013). It was reported that cold stress affects the auxin response pathway primarily through the repression of the auxin transport pathway instead of a signaling pathway, and this effect is linked to the inhibition of intracellular trafficking of a subset of auxin efflux and influx carriers in Arabidopsis (Shibasaki et al. 2009). However, no study reported

159 the role of plant hormone in bamboo responses to cold stress.

160 Cold stress often leads to multiple physiological changes, such as cell membrane
161 damage associated with ion leakage (Whitlow et al. 1992), changes in MDA content
162 (Kong et al. 2016) and proline content (Hayat et al. 2012). Cold stress also stimulates
163 the accumulation of some anti-stress enzymes, such as SOD (Abid et al. 2016; Reddy
164 et al. 2004), POD (Miller et al. 2010) and APX (Caverzan et al. 2012). Evaluating
165 these physiological responses to cold stress in Moso bamboo would effectively
166 determine the effects of cold stress, as well as broaden our understanding of the cold
167 adaptation process in this important species.

168 The draft genome sequence of Moso bamboo was released in 2013 (Peng et al.
169 2013), and an advanced version has recently been mapped at the chromosomal level
170 (Zhao et al. 2018). The draft genomes of four other bamboo species, *Olyra latifolia*,
171 *Raddia guianensis*, *Guadua angustifolia* and *Bonia amplexicaulis* have been
172 published very recently (Guo et al. 2019), providing an excellent opportunity for
173 cold-related studies of this economically and ecologically important grass to be
174 undertaken. The Moso bamboo genome contains 24 *DREB* transcription factors, and
175 *PeDREB1* is strongly induced by cold treatment (Liu et al. 2012; Wu et al. 2015). A
176 recent study revealed that the MYB transcription factor *PheMYB4-1* regulates the cold
177 response in Moso bamboo. Transgenic Arabidopsis plants overexpressing *PheMYB4-1*
178 display increased cold and freezing tolerance, and *PheMYB4-1* may
179 induce *CBF* expression and activate the downstream *COR* genes (Hou et al. 2018). In
180 addition, 13 *TIFY* family transcription factors show up-regulation in response to cold
181 stress (Huang et al. 2016). All these data suggest that the transcriptional regulation is
182 crucial for Moso bamboo's tolerance to low temperature; thus, it is very important to
183 identify the cold-regulated transcription factors in Moso bamboo.

184 In this work, Moso bamboo was used as it is the most common type of bamboo in
185 tropical and subtropical areas. The morphological and physiological changes were
186 recorded after cold treatment; RNA-seq was used to analyze the dynamic changes in
187 transcription that occur at different time points during cold treatment. Two objectives
188 were addressed in this study, namely the identification of candidate genes

participating in cold regulation pathways, and the analysis of expression profiles of key genes involved in cold regulation in Moso bamboo. Overall, our study revealed a broad overview of the Moso bamboo cold-responsive transcriptome, and uncovered cold signal perception and the responsive pathway in Moso bamboo. To the best of our knowledge, this is the first systematic study of the transcriptome profiling of Moso bamboo under cold stress. Our study revealed cold-regulated candidate genes that may potentially be used for generating plants with enhanced cold tolerance.

Material and Methods

Plant material and growth conditions

The seeds of Moso bamboo (*Phyllostachys edulis*) and Ma bamboo (*Dendrocalamus latiflorus* Munro) used in this study were collected from Guangxi Zhuang Autonomous Region (Guangxi, China). Bamboo seeds were thoroughly washed with sterile water and soaked in sterile water for 16 h, and germinated in soil at 22 °C under long-day conditions (16 h of cool white fluorescent light, photon flux of 70 μ mol m^{-2} s^{-1}). For cold treatments, 3-weeks old seedlings at the three-leaf stage were subjected to −2 °C in a freezing chamber (LGX-400B-LED) for 24 h or 72 h, and then allowed to recover at 25°C for 5 days. Seedlings of the control group were grown at 25 °C continuously. Surface structural changes of the abaxial side of the bamboo leaves were imaged using a HITACHI TM3030 PLUS Tabletop Scanning Electron Microscope (SEM) (Hitachi, Japan). To calculate the survival rate, around 30 seedlings were treated with cold for the indicated time, and then allowed to recover for 5 days. The numbers of seedlings alive and dead were calculated and the data were statistically analyzed. All experiments were repeated independently at least 3 times.

Measurement of electrolyte leakage, relative malondialdehyde (MDA) content and superoxide dismutase (SOD), peroxidase (POD), and ascorbic acid peroxidase (APX) activities

Measurement of electrolyte leakage was performed as described previously with

some modifications (Duan et al., 2017). Briefly, leaves were detached from the cold-treated plants and immersed in 50 mL tubes containing 30 mL water, and then the conductivities were measured immediately (S0) with an electrical conductivity meter (type starter 300C, OHAUS, America). The samples were collected after shaking at 120 rpm for 15 min in a vacuum condition, and the conductivities (S1) were determined. Subsequently, the samples were boiled in a water bath with agitation at 120 rpm for 15 min, and the conductivities were measured again after cooling to 25 ℃ (S2). The relative electrolytic leakage (%) was calculated as (S1-S0)/(S2-S0)×100.

All the antioxidant enzymes were measured based on the protocol reported previously with some modifications (Ara et al. 2013). For each sample, five bamboo whole seedlings were pooled together for analysis. For MDA content measurement, whole seedlings (around 0.1 g) were homogenized and mixed with 1 mL MDA reaction buffer consisting of 0.5 % (v/v) thiobarbituric acid and 20 % (v/v) trichloroacetic acid. The mixture was incubated in a water bath at 100 °C for 30 min, and then the reaction was stopped in an ice bath. The mixture was then centrifuged at 10,000 g for 10 min, and the absorbance of the supernatant was measured at 450 nm, 532nm, and 600 nm respectively. The MDA content was calculated based on the protocol of the MDA content Assay Kit (Solarbio, China). For the measurement of the activities of SOD, POD, and APX, Moso bamboo seedlings (0.1 g) were homogenized thoroughly in 50 mmol potassium phosphate buffer (pH 7.8) containing 1 % polyvinylpyrrolidone. The homogenate was centrifuged at 13,000 g for 20 min at 4 °C. The activities of those enzymes were measured using a SOD activity Assay Kit (Solarbio, China), POD activity Assay Kit (Solarbio, China) and APX activity Assay Kit (Solarbio, China), respectively according to the manufacturers' instructions.

Determination of proline (Pro) content

Proline content in Moso bamboo seedling was measured by sulfosalicylic acid-acid ninhydrin method using Pro content Assay Kit (Solarbio, China) (Abraham et al. 2010). Briefly, around 0.1 g of tissues were boiled in 1 mL of 3% sulphosalicylic acid

248 at 95 ℃ for 15 min. The homogenate was centrifuged at 10,000 g for 10 min. About
249 0.5 mL of supernatant was transferred to a new tube containing 0.5 mL of acetic acid
250 and 0.5 mL of acidified ninhydrin reagent. After 30 min of incubation at 95 ℃,
251 samples were kept at room temperature for 30 min and 1 mL of toluene was added to
252 the samples, which were then shaken at 150 rpm to extract red products. The
253 absorbance of the toluene layer was determined at 520 nm using a spectrophotometer.
254 The Pro content was calculated following the manufacturer's instructions (Solarbio,
255 China).
256
257 **Library preparation and transcriptome sequencing**
258 A total amount of 1 μg RNA from each sample was used for sample preparations.
259 Sequencing libraries were generated using a NEBNext® UltraTM RNA Library Prep
260 Kit for Illumina® (NEB, USA) and following the manufacturer's instructions. Index
261 codes were added to attribute sequences to each sample. Briefly, mRNA was purified
262 from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was
263 carried out using divalent cations under elevated temperatures in NEB Next First
264 Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using the
265 random hexamer primer and M-MuLV Reverse Transcriptase. Second strand cDNA
266 synthesis was subsequently performed using DNA Polymerase I and RNase H.
267 Remaining overhangs were converted into blunt ends via exonuclease/polymerase
268 activities. After adenylation of 3' ends of DNA fragments, NEB Next Adaptor with
269 hairpin loop structure were ligated to prepare for hybridization. To select cDNA
270 fragments of preferentially 240 bp in length, the library fragments were purified using
271 the AMPure XP system (Beckman Coulter, Beverly, USA). 3 μL USER Enzyme
272 (NEB, USA) was incubated with the size-selected, adaptor-ligated cDNA at 37°C for
273 15 min followed by 5 min at 95°C before the PCR was started. PCR was performed
274 with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X)
275 Primer. Ultimately, PCR products were purified (AMPure XP system) and library
276 quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering
277 generation of the index-coded samples was performed on a cBot Cluster Generation

System using TruSeq PE Cluster Kit v4-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and paired-end reads were generated.

Transcriptome sequencing

Raw data (raw reads) of fastq format were initially processed through in-house perl scripts. In this step, clean data were obtained from the raw data by removing reads containing adapters, ploy-N regions and low quality reads from raw data. These clean reads were then mapped to the *P.edulis* genome sequence as a reference. Only reads with a perfect match or one mismatch were further analyzed and annotated based on the reference genome. Hisat2 tools were used in mapping with the reference genome (Pertea et al. 2016). At the same time, Q20, Q30, GC-content and sequence duplication levels of the clean data were calculated. All the downstream analyses were based on clean data with high quality. The Fragments per kilobase of transcript per million fragments mapped (FPKM) of each gene was calculated based on the length of the gene and the read counts mapped to the gene.

Differential expression analysis of two time points were performed using the DESeq2 package version 1.22.2 (Love et al. 2014). The resulting *p* values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *p*-value ≤ 0.05 and a fold change (FC) ≥ 1.5 found by DESeq2 were assigned as differentially expressed.

Gene functional annotation

Gene function was annotated based on the following databases: Swiss-Prot (Boeckmann et al. 2003), EuKaryotic Orthologous Groups (KOG) (Tatusov et al. 2000), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Minoru et al. 2008). GO enrichment analysis of the DEGs was implemented by the GOseq R packages based on Wallenius non-central hyper-geometric distribution (Young et al.

2010), which can adjust for gene length bias in DEGs. DIAMOND software (version 0.9.22，https://github.com/bbuchfink/diamond) was used to align the DEGs to the proteins in KEGG, which is a compendium of databases covering both annotated genomes and protein interaction networks for all sequenced organisms. KEGG pathway is part of KEGG database, and is a compilation of manually verified pathway maps to categorize gene functions with the emphasis on biochemical pathways (Minoru et al. 2008). The output of plant-specific KEGG pathways were populated with the KEGG Orthology (KO) assignments in this study.

RNA extraction and qPCR

Total RNA was extracted using the Plant RNA Kit (OMEGA) and reverse transcription using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer's instructions. The quantitative real-time PCR (qPCR) was performed using the TB Green PCR Master Mix Kit (TaKaRa, Japan). The relative expression levels were calculated as described (Huang et al. 2010), and the specific primers for selected 14 DEGs including *PeCML* (*PH01000133G0880*), *CBL-interacting protein kinase* (*PeCIPK1*, *PH01000445G0310*), *cold-responsive protein kinase* (*PeCRPK1*, *PH01000300G0810*), *PeMKK4* (*PH01003465G0120*), *PeMPK3* (*PH01000033G1790*), *PeICE1* (*PH01001045G0070*), *PeMYB15* (*PH01001287G0090*), *PeZAT12* (*PH01001038G0420*), the *phytochrome-interacting factor 3* (*PePIF3* , *PH01000595G0290*), a MYB family transcription factor *REVEILLE1* (*PeREV1*, *PH01000160G0940*), *PeWRKY40* (*PH01001777G0070*) , *PeCBF3* (*PH01000842G0220*), *PeCBF4* (PH01001480G0400), and *PeCOR47* (PH01000447G0290), are listed in Supplemental Table 26. The expression of the housekeeping gene *PeUBQ (PH01000093G1330)* in Moso bamboo was used as internal control as reported previously (Fan et al. 2013).

Results

Effects of cold on Moso bamboo

To assess the effects of cold on Moso bamboo, 3-week-old bamboo seedlings were treated under cold conditions as we described in the material and methods section. Our results showed that the initial wilting and curling of leaves appeared at 24 h and the freezing injury symptoms became more severe at 72 h **(Figure 1a, upper panel)**. After cold treatment, seedlings were transferred to 25 °C and allowed to recover for 5 days. Bamboo seedlings recovered after 24 h cold treatment had reduced leaf expansion and leaf wilting phenotypes, while seedlings recovered from 72 h- cold treatment almost entirely lacking in the chlorophyll and the tissues began wilting-to-death **(Figure 1a, lower panel)**. The mortality ratio after 5 days' recovery was 39 % for 24 h- cold treated plants and 69 % for 72 h- cold treated plants respectively **(Supplemental Figure 1)**. Results from scanning electron microscopy (SEM) clearly demonstrated the collapsed and shrunken trichomes on the abaxial side of the bamboo leaves after 24 h freezing treatment, which were even more obvious after 72 h **(Figure 1b)**.

To evaluate the cold-induced phenotypes, several abiotic stress related to the biochemical parameters were measured. Electrolyte leakage reflects the degree of membrane dysfunction caused by stress, and the increased conductivity is indicative of more severe membrane damage (Whitlow et al. 1992). Our results showed the relative electrolyte leakage of bamboo was increased dramatically with the progression of cold treatment **(Figure 1c)**. The MDA content exhibited a significant increase after 24 h- and 72 h- cold treatment **(Figure 1c)**. SOD, POD and APX work as crucial enzymatic antioxidants to detoxify ROS (Abid et al. 2016; Reddy et al. 2004), and those antioxidant enzyme activities increased significantly after 24 h- and 72 h- cold treatment **(Figure 1c)**. The proline content significantly increased at 24 h and was maintained at a high level at 72 h **(Figure 1c)**. Results from the morphological physiological observations and biochemical assays showed that cold stress at -2 ℃ was detrimental to Moso bamboo, and also indicated that the broad change in gene expression happen within 24 h.

Characterization of the cold-treated Moso bamboo transcriptome

To provide a comprehensive profile of the transcriptome of Moso bamboo in response to cold, we performed RNA-Seq analysis. To optimize the conditions for this experiment, bamboo seedlings treated at -2 ℃ were harvested at different time points (0 h，0.5 h, 1 h, 3 h, 6 h, 12 h, and 24 h) to analyze the expression patterns of several cold responsive marker genes such as *PeCRPK1*, *PeCML*, *PeCBF3*, *PeMPK3*, *PeZAT12*, *PePIF3*, *PeMKK4*, *PeICE1*, and *PeCOR47* (Kidokoro et al. 2017; Pareek et al. 2017; Shi et al. 2015). Our results showed that in most cases, these genes were responsive at 0.5 h or 1 h, and had the most significant change at 24 h **(Supplemental Figure 2)**. Therefore, samples from cold treatment under -2 °C at 0 h, 0.5 h, 1h and 24 h were used for RNA sequencing. A total of 12 samples, including three biological duplicates at each of the four time points were performed.

Illumina platform generated 269,435,030 raw reads. After filtering, 266,080,018 clean reads containing a total of 79.27 Gb clean nucleotides with 91.68% Q30 bases (base quality > 30) were obtained through stringent quality assessment and data filtering. The quality of the sequencing data is summarized in **Supplemental Table 1**. The clean reads were mapped to the *P.edulis* genome using the HISAT2 tool. The average mapping ratio ranged from 85.21% to 89.35%. Based on the read alignments, StringTie was applied to transcript assembly (Pertea et al. 2015). After optimal gene structure prediction and alternative splicing analysis, a total of 47,092 genes were identified, with 15,105 (32.1%) new genes.

To validate and annotate the assembled transcriptome library, we searched against the Non-redundant (Nr) peptide database, Swiss-Prot protein database, KOG and KEGG, using BLASTx with a cutoff E-value of 10^{-5}. The results indicated that over 65% of the transcripts had significant similarity to at least one target from these databases **(Supplemental Table 2)**.

Global change of the cold-responding transcripts in Moso bamboo

The DEGs were determined as cold-responsive genes if the fold change in expression levels was at least 1.5 fold change and the adjusted *p*-value $\leqslant$ 0.05 at any time point compared to control using DESeq2. The DEGs identified at 0.5 h and 1 h

were defined as early responsive genes, and those that changed exclusively at 24 h were regarded as the late responsive genes.

In total, 2,463 DEGs which cover 5.2% of all Moso bamboo genes were identified under cold treatment, of which, 1,177 (47.8%) were up-regulated **(Figure 2a, Supplemental Table 3)** and 1,286 (52.2%) were down-regulated **(Figure 2a, Supplemental Table 4)**. Our results demonstrated that 73 and 59 genes were up-regulated at 0.5 h or 1 h respectively while 1,137 genes were up-regulated at 24 h **(Supplemental Table 5, 6 and 7)**. Among all the cold up-regulated genes, only 26 genes had increased expression levels at all the time points **(Supplemental Table 8)**. In the down-regulated category, only 16 genes were down-regulated at 0.5 h and 20 genes at 1 h, while 1,263 genes were down-regulated at 24 h **(Supplemental Table 9, 10 and 11)**, with only 2 genes showing decreased expression at all time points **(Supplemental Table 12)**. The expressions levels of 1,072 up-regulated and 1,253 down-regulated genes were completely changed at 24 h. The comparison of the number of early responding genes (132 up-regulated and 36 down-regulated genes) suggested that the observed gene up-regulation may play a key role in the early response to cold stress.

To visualize the expression patterns of these DEGs at early and late stages, a heatmap was constructed on the basis of the fragments per kilobase of transcript per million (FPKM) values **(Figure 2b)**. DEGs with similar expression patterns were grouped, and the heatmap results showed that most DEGs changed their expression profile significantly at 24 h. Our data suggested that the majority of the cold-regulated genes are late-response genes under our treatment conditions. This observation in bamboo is similar to the previous reports in Arabidopsis which showed that most induced or repressed genes appeared at 24 h cold treatment (Lee et al. 2005).

To further verify the RNA-seq data, we performed qPCR analysis for 12 selected DEGs that are known to be related to cold stress, including *PeCML*, *PeCIPK1*, *PeCRPK1*, *PeMKK4*, *PeMPK3*, *PeICE1*, *PeMYB15*, *PeZAT12*, *PePIF3*, *PeREV1*, *PeWRKY40* and *PeCBF3*. The transcripts of those 12 genes showed similar

expression patterns to the results from RNA-seq **(Figure 2c)**. These results support the validity of the Moso bamboo cold-regulated transcriptome from the *in silico* analysis.

Functional annotation and classification of cold-regulated genes

To characterize the functional classifications of the cold-regulated genes, 77.03 % of up-regulated transcripts and 67.86 % of down-regulated transcripts were matched to the Gene Ontology (GO) database, resulting in three categories being identified: biological processes, cellular components, and molecular functions **(Supplemental Figure 3a and 3b)**. In each of the three main GO classifications, "Cellular process", "Cell" and "Binding" exhibited the highest match numbers among up-regulated and down-regulated genes **(Supplemental Table 13 and 14)**. We noticed a higher percentage of genes from the "Rhythmic process" and "Positive regulation of biological process" existed in the up-regulated genes compared with down-regulated genes **(Supplemental Figure 3, Supplemental Table 13)**. "Cell killing" and "Detoxification" genes were only present among the up-regulated genes. On the other hand, the "Growth" and "Cellular component organization or biogenesis" groups were more abundant amongst down-regulated genes **(Supplemental Figure 3, Supplemental Table 13 and 14)**. This data suggested that cold stress might provoke the expressions of genes involved in cell killing and detoxification processes, which is consistent with the phenotypes observed and shown in **Figure 1**.

For further functional prediction and categorization, all cold-regulated genes were subject to phylogenetic classification using the KOG database. The up-regulated and down-regulated genes were assorted in 22 and 24 KOG categories, respectively **(Supplemental Figure 4)**. "Signal transduction mechanisms", "General function prediction only", and "Posttranslational modification" genes were the most represented categories **(Supplemental Table 15 and 16)**. "Secondary metabolites biosynthesis", "Energy production and conversion", and "Carbohydrate transport and metabolism" were over-represented amongst the up-regulated genes compared to down-regulated genes **(Supplemental Table 15)**, which implied that genes involved

in carbohydrate and secondary metabolism pathways might contribute to cold resistance processes. “Chromatin structure and dynamics”, “Replication, recombination and repair” and “Cell cycle control, cell division, chromosome partitioning” were more abundant in down-regulated genes compared to up-regulated genes **(Supplemental Table 16)**. In addition, “Nucleotide transport and metabolism” and “Extracellular structures” were only identified among down-regulated genes **(Supplemental Figure 4b, Supplemental Table 16)**. These findings suggested that in bamboo chromatin remodeling was crucial in regulating cold response, and also hinted that the genes involved in the repair process and cell division were heavily inhibited in response to cold stress.

To identify candidate metabolic pathways regulated by cold stress, the DEGs were examined using the KEGG pathway analysis tool, which is a compilation of manually verified pathway maps to categorize gene functions with the emphasis on biochemical pathways (Minoru et al. 2008). A total of 65.23% up-regulated genes and 58.03% down-regulated genes were mapped to the KEGG database. For both the up- and down- regulated genes, the clusters for “Metabolism” and“Organismal systems” were significantly enriched **(Supplemental Figure 5)**. The cold-regulated genes were further classified into five categories **(Figure 3)**. Interestingly, within the “Metabolism” category, “Global and overview maps”, “Carbohydrate metabolism” and “Amino acid metabolism” genes were most highly represented amongst the up-regulated genes **(Figure 3a)**. Several genes encoding enzymes associated with cysteine and methionine metabolism, including S-adenosylmethionine synthetase, cystathionine gamma-synthase and adenosylmethionine decarboxylase were specifically induced by cold stress **(Supplemental Table 17)**. Moreover, a few genes involved in flavonoid biosynthesis, including phenylalanine ammonia-lyase and chalcone synthase were significantly increased, suggesting that Moso bamboo may use flavonoids as antioxidants to prevent from ROS damage **(Supplemental Table 17)**. Pathways related to “Environmental adaptation” were highly represented in up-regulated genes **(Figure 3a)**, with calcium- binding proteins being especially notable **(Supplemental Table 17)**. These findings indicated a role of calcium- binding

proteins as the main signaling components in cold signal transduction. Furthermore, "Membrane transport" composed of ABC transporters were mainly represented in up-regulated genes **(Figure 3a; Supplemental Table 17)**. Several ABC transporters were rapidly induced in Moso bamboo after cold exposure **(Supplemental Table 17)**. On the other hand, "Nucleotide metabolism", "Replication and repair", "Glycan biosynthesis and metabolism" and "Lipid metabolism" were down-regulated more during cold stress **(Figure 3b)**. Many genes related to cell wall modification were found in the category of "Glycan biosynthesis and metabolism" **(Supplemental Table 18)**. Meanwhile, the genes involved in 'Biosynthesis of unsaturated fatty acids' and 'Fatty acid elongation' were significantly inhibited by freezing treatment **(Supplemental Table 18)**, indicating a decrease in fatty acid content in the plasma membrane.

Expression profiles of cold-regulated genes

The heatmap demonstrated that dynamic transcriptional changes in response to the cold stress **(Figure 2b)**. The identified DEGs were grouped into 12 clusters based on the SOM cluster analysis using the *k*-means method **(Figure 4, Supplemental Table 19)**. The KOG functional category was applied for each cluster to predict the distribution of different functions among the three time periods. The most abundant cluster was Cluster 10 with 573 DEGs induced immediately at 0.5 h and remained up-regulated at 24h. The second most abundant cluster was Cluster 8, which was comprised of 373 genes with significantly increased expression at 24 h. The third most abundant group was Cluster 5, containing 292 genes with decreased expression at 0.5 h and 24 h. The fourth most abundant group was Cluster 3 with 263 genes showing decreased expression at 24 h **(Supplemental Table 19)**.

Cluster 6 with 164 genes was rapidly induced at 0.5 h but not at later time points, while cluster 1, 4 and 10 showed another peak at 24 h **(Figure 4)**. Interestingly, functional categories of "Transcription", "Lipid transport and metabolism" and "Inorganic ion transport and metabolism" were over-represented in Clusters 1, 6 and 10 **(Supplemental Table 20)**. This indicated that cells received the cold signal and

instantly transmitted through the ion and lipid transport through transcription network within 0.5-1 h cold treatment. The genes with functions of "Amino acid transport and metabolism", "Carbohydrate transport and metabolism", and "Energy production and conversion" were identified as up-regulated throughout the 1 - 24 h stage **(Figure 4, Supplemental Table 20)**, reflecting the downstream metabolic processes activated by signal transduction and transcription in response to cold. Clusters 2, 3, 5, 7 and 11 contained 1234 down-regulated genes at 24 h, while cluster 2, 5 and 7 responded to the cold stress rapidly in the early stage (0-0.5 h) by decreasing their expression **(Figure 4)**. Functional categories of "Nucleotide transport metabolism", "Chromatin structure and dynamics", "RNA processing and modification" were more enriched in those clusters **(Figure 4, Supplemental Table 20)**, as shown by those processes appearing to be rapidly negatively regulated by cold stress. In summary, our results indicated that genes responded to cold stress in a hierarchical manner in bamboo.

Transcription factors responding to cold stress

Transcription factors play important roles in mediating cold stress related gene expressions (Lee et al. 2005; Zhang et al. 2014). In this study, we identified 222 transcription factors from 24 different families, which were differentially expressed throughout the 24 h cold stress **(Figure 5a; Supplemental Table 21)**. A total of 111 up-regulated transcription factors were identified from 19 different families/groups **(Figure 5b, Supplemental Table 22)**. The most up-regulated transcription factors constituted key families that are cold-sensitive, such as APETALA2, ethylene response factors (AP2/ERF), WRKY transcription factors (WRKY), NAC domain-containing proteins (NAC), and basic leucine zipper transcription factors (bZIP) **(Figure 5b, Supplemental Table 22)**. The number of down-regulated transcription factors was comparable to that of up-regulated transcription factors, which consisted of 111 genes from 22 families, which were mainly from MYB, homeodomain-leucine zipper transcription factor (HD-ZIP), and B3 domain-containing transcription factor (or B3) families **(Figure 5b, Supplemental Table 23)**.

The expression changes of cold responsive transcription factors were illustrated by heatmap analysis, and those with similar expression patterns were categorized **(Figure 5c)**. Some transcription factors were induced immediately after the plants were exposed to cold stress, while others were down/up-regulated subsequently, suggesting that a transcriptional cascade triggered by cold stress might be present in Moso bamboo. The analysis highlighted the expression changes of several well-known cold-regulated transcription factors during 24 h cold treatment. For example, *PeCBF3* and *PeWRKY33* responded rapidly to cold treatment at 0.5 h, and their expression levels also increased again at 24 h. The expression level of *PeREV1* and *PeMYB15* increased at 24 h **(Supplemental Table 21)**. In addition, the expression of the *PeWRKY40* and *PeZAT12* increased immediately at 0.5 h and maintained a positive slope until 24 h cold treatment **(Supplemental Table 21)**, which is consistent with a previous finding that these proteins serve as the markers for early cold response in Arabidopsis (Lee et al. 2005).

Expression patterns of selected DEGs in two bamboo species with different cold tolerant abilities

Since no Moso bamboo genetic transformation method was available at the time, we could not verify the function of DEGs from our RNA seq results through gene modification method. Alternatively, the gene function might be deduced by comparing the expression patterns in different bamboo populations with different cold-tolerance abilities. Ma bamboo (*Dendrocalamus latiflorus Munro*) is a more cold-sensitive bamboo species compared to Moso bamboo (Liu et al. 2006). To examine the expression patterns of the cold stress-induced genes from RNA seq data, Ma bamboo and Moso bamboo were exposed to low temperature over 24 h and the gene expression patterns were examined by qPCR. Six representative DEGs including two putative positive regulators (*PeCBF3* and *PeCBF4*) and four putative negative regulators (*PeMYB15*, *PePIF3*, *PeZAT12*, and *PeCRPK1*) in cold signaling pathways were selected for this experiment. Our results demonstrated that all genes tested were cold responsive **(Supplemental Figure 6).** More importantly, compared with the less

cold-tolerant Ma bamboo, Moso bamboo has higher expression levels of putative positive regulators and lower expressions of negative regulators in cold signaling pathway **(Supplemental Figure 6)**. These data reflected the effectiveness of our RNA-Seq results, and support functional significance of DEGs in bamboo cold signaling pathways.

Discussion

Calcium signaling pathway and MAPK cascades were activated in the early stage of the bamboo cold response

We noticed that various Ca^{2+} sensor genes including *PeCaM/CP1*, *PeCML*, *PeCDPKs* (*PeCDPK19* and *PeCDPK5*), *PeCRPK1*, and *PeCIPK1* were significantly induced in response to cold stimulus **(Supplemental Table 17)**. Our data also indicated that genes encoding Ca^{2+} binding proteins responded to cold treatment within 30 min **(Figure 2c)**, which was consistent with previous findings that Ca^{2+} binding proteins rapidly transduced external signals (Kudla et al. 2018). Moreover, our data demonstrated that the *MAPK* cascades, such as *PeMKK9* (*PH01003362G0140*), *PeMKK4*, *PeMPK20* (*PH01000298G0100*), and *PeMPK3* were activated in the early stage of cold treatment **(Figure 2c and Supplemental Table 17)**.These data supported previous findings that the activation of the *MAPK* cascade could be triggered by cold stress, probably due to the over-accumulation of ROS and MDA content (Zhang et al. 2014).

Our data supported the hypothesis that Ca^{2+} and *MAPK* signal transduction pathways were activated at an early stage when bamboo was challenged with cold stress to induce downstream gene expression and protect the plant cells. The data are consistent with previous results from Arabidopsis and rice (Lee et al. 2005; Zhang et al. 2014). Moreover, we also noticed that several important negative regulators in Arabidopsis or rice might play opposite roles in bamboo during cold response. For example, it was reported that Arabidopsis *CRPK1* functions as a negative regulator through the *CBF* pathway (Liu et al. 2017), and another key regulator, *MPK3*,

negatively regulates *ICE1* expression through post-translational modification (Li et al. 2017). Interestingly, the expressions of both orthologs in Moso bamboo were enhanced in response to cold treatment, indicating the presence of the bamboo species-specific control mechanisms in response to cold stress.

Transcription factors response to cold stress in both plant kingdom-conserved and species- specific mechanisms in Moso bamboo

A large number of transcription factors belonging to different transcription factor families have been shown to play a crucial role in regulating the cold response in Arabidopsis (Lee et al. 2005), rice (Zhang et al. 2014), wheat and many other plant species (Calzadilla et al. 2016; Kargiotidou et al. 2010; Wang et al. 2014; Weiss and Egea-Cortines 2009). Here, we identified 222 transcription factors from 24 different gene families responding to cold stress. Among them, MYB, AP2/ERF, WRKY, ZIP families comprise a high proportion of cold-responsive members **(Figure 5a)**. We investigated the classical *CBF* regulation pathway including the upstream regulators, such as *PeICE1*, *PeMYB15*, *PeZAT12* and *PePIF3*. Interestingly, *PeICE1*, *PeZAT12*, *PeMYB15* and *PePIF3* were induced rapidly by cold stress at 0.5 h **(Figure 2c)**, which demonstrated the effectiveness of our treatments and confirmed the important roles of transcription factors in the early cold response in bamboo. *ICE1* is a key positive regulator of *CBF3* (Chinnusamy et al. 2003), while *ZAT12*, *MYB15* and *PIF3* are all negative regulators of *CBF* genes (Agarwal et al. 2006; Jiang et al. 2017; Novillo et al. 2007). The combination of the regulation of *PeICE1*, *PeZAT12*, *PeMYB15* and *PePIF3* could explain the fluctuation of *PeCBF3* expression. Previous studies showed that 8 *WRKYs* display increased expression in the early cold respond response in Arabidopsis (Lee et al. 2005). We found that the enhanced expression of two *PeWRKYs* (*PeWRKY40* and *PeWRKY33*) occurred at an early stage in response to cold stress in Moso bamboo **(Figure 2c and Supplemental Table 21)**, which suggested that the *PeWRKYs* might have conserved roles in the cold response in bamboo. We also identified *PeREV1*, whose ortholog in Arabidopsis works as a negative regulator of cold acclimation (Meissner et al. 2013), showing increased

expression during the cold treatment. The expression patterns of important regulators of the *CBF* pathway could explain the cold-sensitive phenotypes of Moso bamboo via the repression of *PeCBF3*.

It is worth noting that except for the *CBF* pathway, other cold stress regulatory pathways also play a role in plant cold response (Fowler and Thomashow 2002; Kreps et al. 2002; Monroy et al. 2007; Tian et al. 2013). For example, it was reported that at least 28% of the cold-responsive genes were not regulated by the *CBF* pathway in Arabidopsis (Fowler and Thomashow 2002). Furthermore, at least one-third of the cold-inducible genes in wheat were independent of the *CBF* pathway (Monroy et al. 2007). In Moso bamboo, 40 transcription factors were induced immediately at the transcriptional level upon exposure to cold stress **(Supplemental Table 24)**. The early induced transcription factors, which were closely clustered with known transcription factors such as *PeWRKY33*, *PeCBF3* and *PeMYB15*, warrant further investigation to identify a potential *CBF* parallel pathway. For example, the up-regulation of HD-ZIP transcription factor (*PH01001036G0340*), the NAC transcription factor (*PH01001177G0140*) and the B3 transcription factor (*PH01000246G0410*) within 0.5 h by cold stress, indicate that those regulators play an important function in cold stress.

The transcriptomic profiles of cell wall related genes changes in response to cold stress

Plant cell walls play a structural role in plant abiotic stress defenses (Tenhaken 2015). It has been proposed that increasing the amount of pectin could efficiently delay plant cell damage by forming hydrated gels (Leucci et al. 2008). Interestingly, our data revealed that three genes involved in the biosynthesis of pectin, *PeGAE1* (*PH01000119G0710*) encoding a UDP-D-glucuronate 4-epimerase, *PeRHM1* (*PH01001109G0280*) encoding a UDP-L-Rhamnose synthase and *PeGATL9* (*PH01000092G1070*) encoding a galacturonosyl transferase, were highly induced during the cold treatment **(Supplemental Table 17)**. These results suggested that these genes might function not only in cell wall metabolism but also as candidates for

664 the plant adaption to cold stress in Moso bamboo. On the other hand, we showed
665 several key genes affecting cell wall integrity through xylan modification, such as a
666 1,4-beta-D-xylan synthase (*PeIRX10, PH01000002G2800*), two glycosyl transferases
667 (*PeIRX9, PH01000428G0570* and *PeIRX9L, PH01000256G1170*), and a
668 plant-specific *PeDUF231* (*PeTBL27, PH01001319G0100*), were dramatically
669 down-regulated at 24 h **(Supplemental Table 18)**. As xylan is the major component
670 of hemicelluloses in the plant cell wall, reduced expression of xylan biosynthesis
671 genes leads to the weakening of the secondary cell wall, resulting in the collapse of
672 xylem vessels (Brown et al. 2007; Lin et al. 2016). Our data indicates cell wall-related
673 genes play important roles in the acquisition of cold tolerance by changing their
674 expression patterns at the transcription level.
675
676 **Lipid metabolism was inhibited under cold stress**
677 It was documented that the most damaging effect of cold stress in plants is plasma
678 membrane damage from dehydration (Steponkus 1993). The cold-treated bamboo
679 displayed obvious dehydration phenotypes, such as wilting **(Figure 1a)** and ruptured
680 trichomes on the leaf surface **(Figure 1b)**. Damage of the plasma membrane was
681 demonstrated in terms of increased ion leakage, which implies increased membrane
682 permeability and reduced cell tolerance to low temperature **(Figure 1c)**. Furthermore,
683 the increased content of MDA indicated the oxidation of the unsaturated membrane
684 fatty acids **(Figure 1c)**. Increased accumulation of unsaturated fatty acids in the
685 plasma membrane improve cold defense by preventing ion leakage (Degenkolbe et al.
686 2012). Based on the KEGG metabolic pathway analysis, the 'Biosynthesis of
687 unsaturated fatty acids' and 'Fatty acid elongation' clusters were significantly
688 enriched in down-regulated genes **(Supplemental Table 18)**. In particular, genes
689 involved in unsaturated fatty acids biosynthesis including an acyl-CoA dehydratase
690 (*PePAS2, PH01001117G0220*), a stearoyl-ACP desaturase (*PeFAB2,*
691 *PH01001326G0300*), and a NADP-binding protein
692 (Phyllostachys_edulis_newGene_23189) were down-regulated at 24 h **(Supplemental**
693 **Table 18)**. Several genes involved in cuticle membrane and wax biosynthesis, such as

PeCER3 (*PH01000379G0490*), *PeMYB106* (*PH01005515G0070*), *PeKCS4* (3-ketoacyl-CoA synthase, *PH01000046G1090*), *PeKCS5* (*PH01001011G0290*) and *PeKCS6* (*PH01000101G1030*) were also down-regulated at 24 h **(Supplemental Table 18 and 21)**. Our data showed that the decreased expression of genes involved in the biosynthesis of fatty acids in response to cold would explain the impaired cold defense and cold-sensitive phenotypes, which are consistent with previous findings (Degenkolbe et al. 2012; Shepherd and Griffiths 2006).

Phytohormones play important roles in plant cold-stress response

The crucial roles of plant hormones in the plant cold stress response have been well demonstrated (Shi et al. 2015). Of the DEGs we identified, 71 genes were involved in ABA-, ethylene-, GA- and auxin- related pathways **(Supplemental Table 25)**. In cold treated bamboo, expressions of genes involved in ABA biosynthesis, signal reception and downstream signaling pathways were changed. A putative *NCED* gene (*PH01000283G0010*) which encodes a key enzyme in ABA biosynthesis pathway, and a PYR/PYL/RCAR family protein (*PH01002424G0210*), that functions as an ABA sensor, had enhanced expression in the cold-treated bamboo seedlings. In plants, members of the protein phosphatase 2C (PP2C) family may act as positive regulators within ABA-mediated signaling networks activated by diverse environmental stresses or developmental signaling cascades (Xue et al. 2008), with two PP2C family genes (*PH01001115G0280* and *PH01004966G0010*) displaying increased their expression levels after cold treatment in bamboo. A total of 24 putative ABA responsive genes with 17 being up-regulated and 7 being down-regulated were identified in this study **(Supplemental Table 25)**, suggesting that ABA related pathways participated in bamboo cold response. The role of ethylene in plant response to cold is different in different species (Kazan 2015). We noticed the down-regulation of a putative *ETO1* (ethylene over-producer) paralog (*PH01000367G0090*) after cold treatment, which acts as a negative regulator of *ACS5* (1-aminocyclopropane-1-carboxylate synthase 5, a key enzyme in ethylene biosynthesis pathway), indicating the enhanced level of ethylene in bamboo after cold treatment. Ethylene signaling pathway also affects plant

cold tolerance. In cold treated bamboo, at least 12 ethylene responsive factors (ERF) changed their expression patterns, with 9 being up-regulated and 3 being down-regulated **(Supplemental Table 25)**. These results suggest ethylene pathways might be activated during the bamboo cold response. A key response to cold in plants is growth repression, to allow the plant to re-allocate resources from growth to processes responsible for increasing cold tolerance (Eremina et al. 2016). Gibberellins and auxin are well known growth-promoting hormones, and our results indicate their potential roles in the bamboo cold response. In the cold-treated seedlings, one GA 2-oxidase gene (*PH01001124G0470*) which deactivates gibberellins was up-regulated **(Supplemental Table 25)**, indicating GA homeostasis was required under cold stress in bamboo. The growth hormone auxin essentially regulates all aspects of plant developmental processes under both normal and abiotic stress conditions. The effect of cold stress on auxin is linked to the inhibition of intracellular trafficking of auxin efflux carriers (Rahman 2013). In accordance with these findings, the putative auxin influx carrier *PeLAX2* (*PH01000484G0740*) and efflux carrier *PePIN1* (*PH01000484G0740*) had reduced expression levels in bamboo **(Supplemental Table 25)**. Moreover, a putative AGCVIII kinase (*PH01000023G1420*), which positively activates the PIN-mediated auxin efflux by affecting cell trafficking (Willige and Chory 2015), was also down-regulated **(Supplemental Table 25)**. Recently, it was found that abiotic stress-induced growth inhibition involves repression of auxin responsive genes (Shani et al. 2017). Our results align with these findings, since we found that multiple early auxin-responsive genes had altered expression levels in bamboo. For example, a putative transcriptional repressor *AUX/IAA* (*PH01000025G1630*) increased its expression, and 5 auxin-inducible small auxin up RNA genes (*SAUR*) had reduced expression levels **(Supplemental Table 25),** indicating the inhibition of the auxin pathways. Those results suggested that cold stress affected auxin effects mainly through disrupting its transport and signaling pathway in bamboo. In summary, our results indicated that the phytohormone functions in the bamboo cold response, while the detailed precise mechanisms behind this action need to be further investigated.

Hypothetical model occurring in leaves of Moso bamboo upon cold stress

A previous transcriptome study in Arabidopsis indicated that 3.9% of all Arabidopsis genes were cold stress response genes (Lee et al. 2005). The majority (74%) of the cold-response genes were late-response genes, which only displayed altered expression levels only after 24 h of cold treatment. The significant transcriptomic changes at the late stage were related to primary and secondary metabolism and photosynthesis (Lee et al. 2005). The early-response genes were mainly identified as transcription factors and the genes related to hormone biosynthesis and signaling (Lee et al. 2005). A study of transcriptome reprogramming in cold acclimation of tomato indicated that the early changes in expression are mainly associated with transcription factors. In contrast, the late response that took place after 24 h of cold exposure caused changes in expression of genes involved in metabolism and machinery associated with protein translation (Barrero‐Gil et al. 2016). Based on the results presented in this study, we propose a model for the cold signal perception and responsive pathways in Moso bamboo **(Figure 6)**. According to this model, freezing temperatures are rapidly recognized through calcium signaling pathways, *MAPK* cascades and other pathways such as ABA signaling cascades. These signaling pathways stimulate transcriptional reprogramming including CBF-dependent or CBF-independent pathways that subsequently trigger a complex series of metabolic activities, including antioxidant production, cell wall composition adjustment and lipid metabolism alteration. Notably, several negative regulators of cold tolerance, such as *PeREV1*, *PePIF3*, *PeMYB15* and *PeZAT12* were effectively up-regulated during the early stage of cold stress. This specific expression pattern is speculated to be responsible for the cold-sensitive phenotypes of Moso bamboo. The findings in this study will contribute to the elucidation of the molecular mechanisms underlying the low-temperature response, which could significantly contribute to improving cold tolerance in Moso bamboo.

Conclusion

In this study, we demonstrated the physiological and biochemical changes that occur in Moso bamboo in response to cold stress. The genome-wide transcriptome analysis shed light on the DEGs involved in cold regulation. We found that the Ca^{2+} signaling pathway and MAPK cascades responded rapidly to cold stress. Additionally, transcription factors involved in the key signaling pathways in response to cold stress were identified in this study. Moreover, our results demonstrated that the expression of genes involved in various metabolism pathways, such as secondary metabolites biosynthesis and lipid metabolism, were altered during cold treatment, revealing the potential role of these genes in cold defense. The results from this study provided information to further elucidate of the possible functions of cold responsive genes in bamboo. In the future, more experimental and bioinformatics work will be needed to reveal the functions of these important candidates in this important species.

Availability of data and materials

RNA-Seq in this study had been submitted to GEO under accession number GSE130314.

Supplemental Material

Supplemental Figures and Tables are listed

Conflict of interests

The authors declare that they have no conflict of interests.

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Authors' contributions

Q.Z., and Y.L. conceived this project; C.T.L., Y.S.Z., Y.L. and Q.Z. designed experiments and interpreted the results. Y.L., C.W., H.X. and H.G. performed the experiments; Y.W., S.C., H.L., and G.W.L. helped to collect and analyze the data. Y.L. and Q.Z. wrote the manuscript. All authors read and approved the submission of this manuscript.

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Figure 1. **Effects of freezing stress on Moso bamboo seedlings**

(a) Upper Panel: Morphological changes of bamboo seedlings after cold treatment. 3-week-old Moso bamboo seedlings that were exposed to -2°C for 24 h and 72 h respectively. Pictures highlighted in boxes show a closer view of the unstressed and freezing stress-exposed leaves. Bars = 1cm;

Lower Panel: Morphological changes of bamboo seedlings recovered after cold treatment. 3-week-old Moso bamboo seedlings were exposed to -2°C for 24 h or 72 h, and then allowed to recover at normal growth temperatures for 5 days. Pictures highlighted in boxes show a closer view of the unstressed and freezing stress-exposed leaves. Bars = 1cm;

(b) Scanning electron microscopy images of the lower surfaces of Moso bamboo leaves showing the collapse of trichomes due to the freezing treatment. The lower panel shows higher magnification images of the red boxed area in upper panel. Arrows indicate ruptured trichomes. Bars = 100 μm.

(c) Measurements of physiological and biochemical parameters reflecting damage of Moso bamboo leaves. Values are means from three replications and error bars represent the standard deviations. Asterisks indicate significant differences from 24 h and 72 h to 0 h based on Student's t test data. Statistically significant differences were indicated by: *, $p<0.05$, **, $p<0.01$. All the measurements were performed at least three times with similar results and representative data from one repetition were shown.

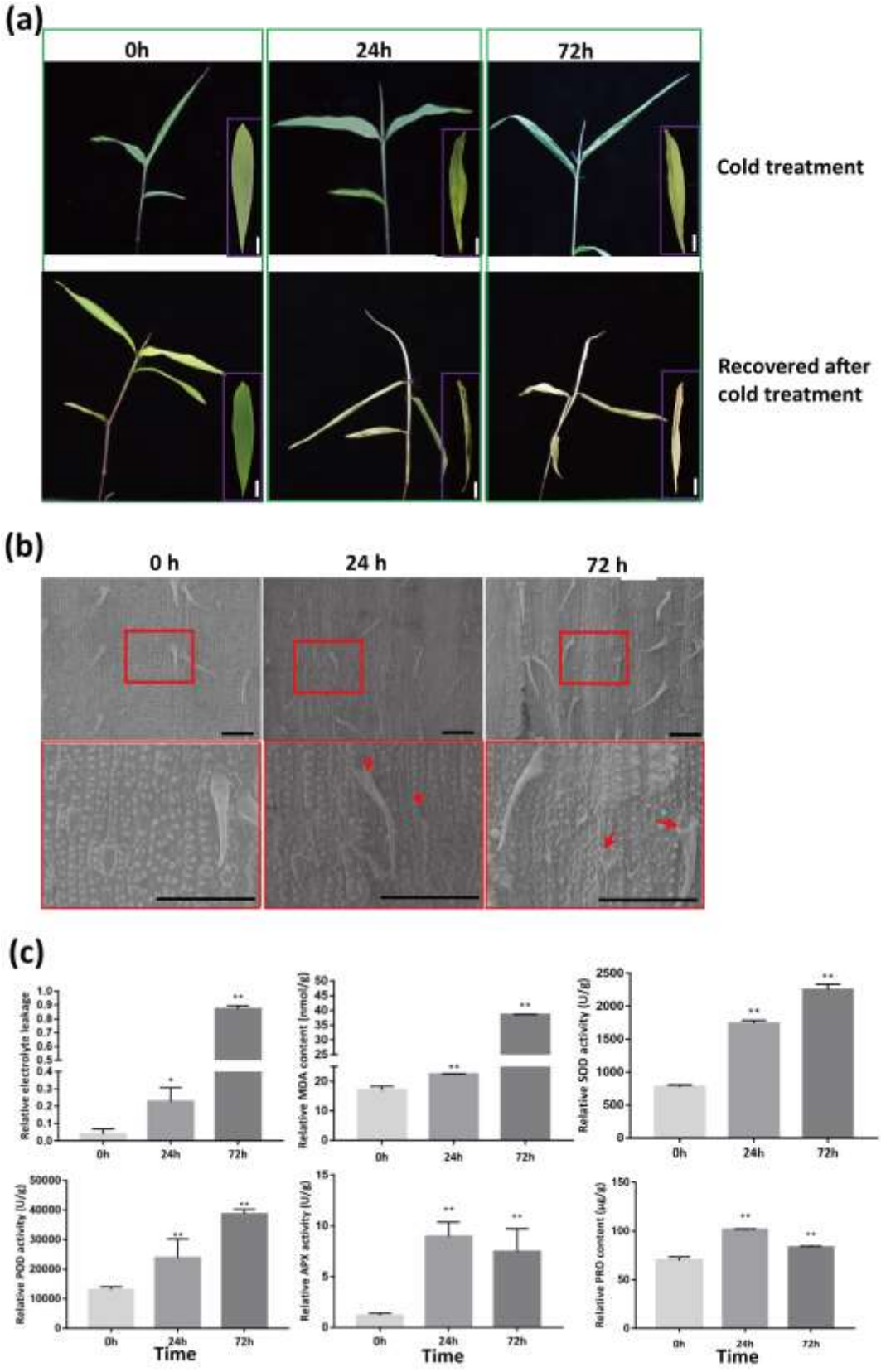

Figure 2. Overview of the differentially expressed genes in response to cold stress in Moso bamboo

(a) Venn diagrams of cold-regulated genes. Figures in rectangles indicate cold treatment hours (h) and total number of cold-regulated genes at each time point.

(b) Heat map of RNA-Seq transcriptome analysis for 2463 DEGs. Columns and rows in the heat map represent samples and genes, respectively. Sample names are displayed below the heat maps. The color bar is the scale for the expression levels of each gene. (c) Real-time PCR analysis of 12 selected genes in Moso bamboo. Data represents the average of three independent experiments ± Standard Error (SE).

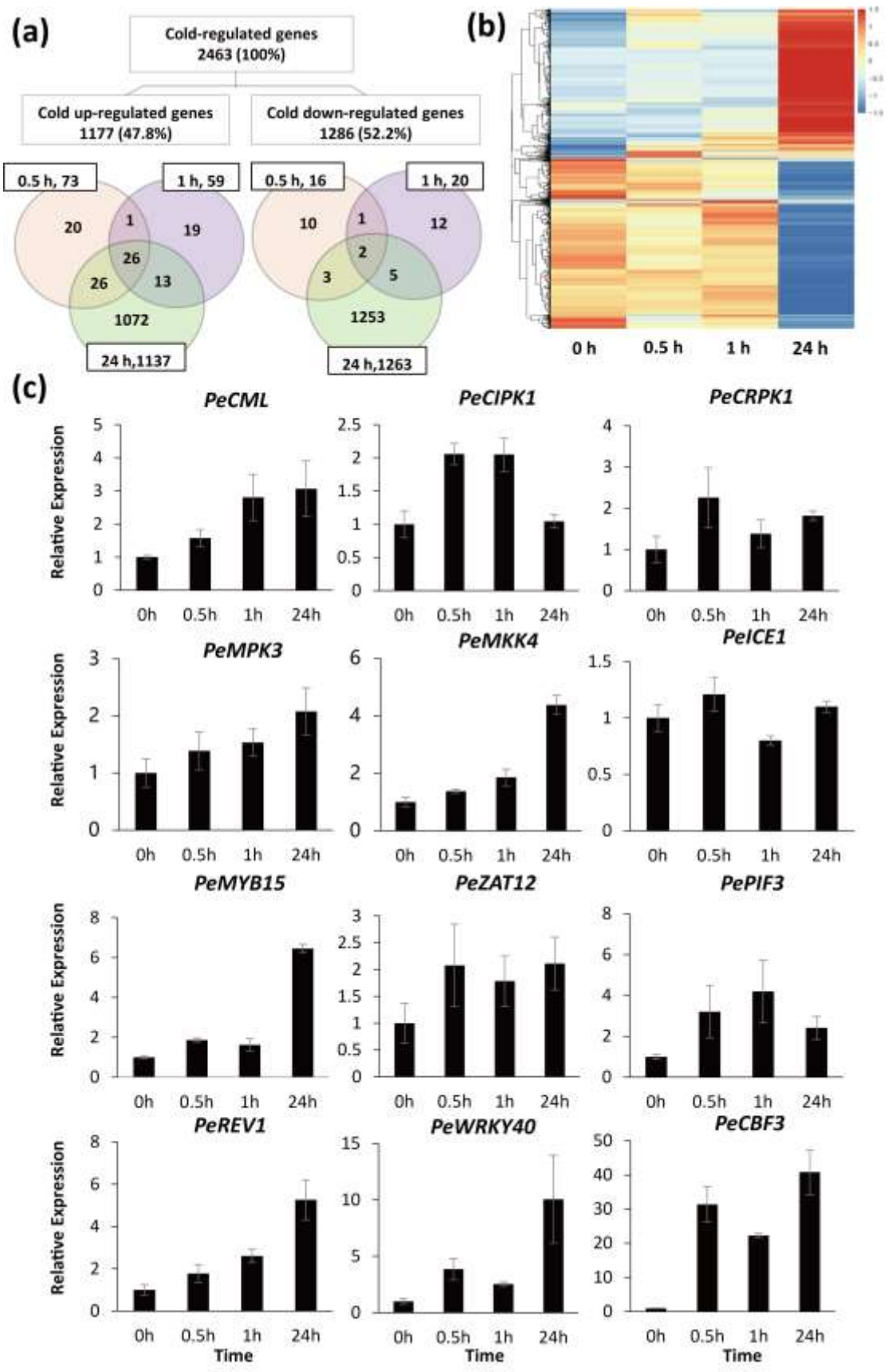

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Figure 3. Classification of Moso bamboo cold stress responsive genes for each KEGG category

The results are summarized in five categories: Cellular processes, Environment information processing, Genetic information processing, Metabolism and Organismal systems. The x-axis indicates the number of genes in a category.

(a) Up-regulated genes; (b) Down-regulated genes.

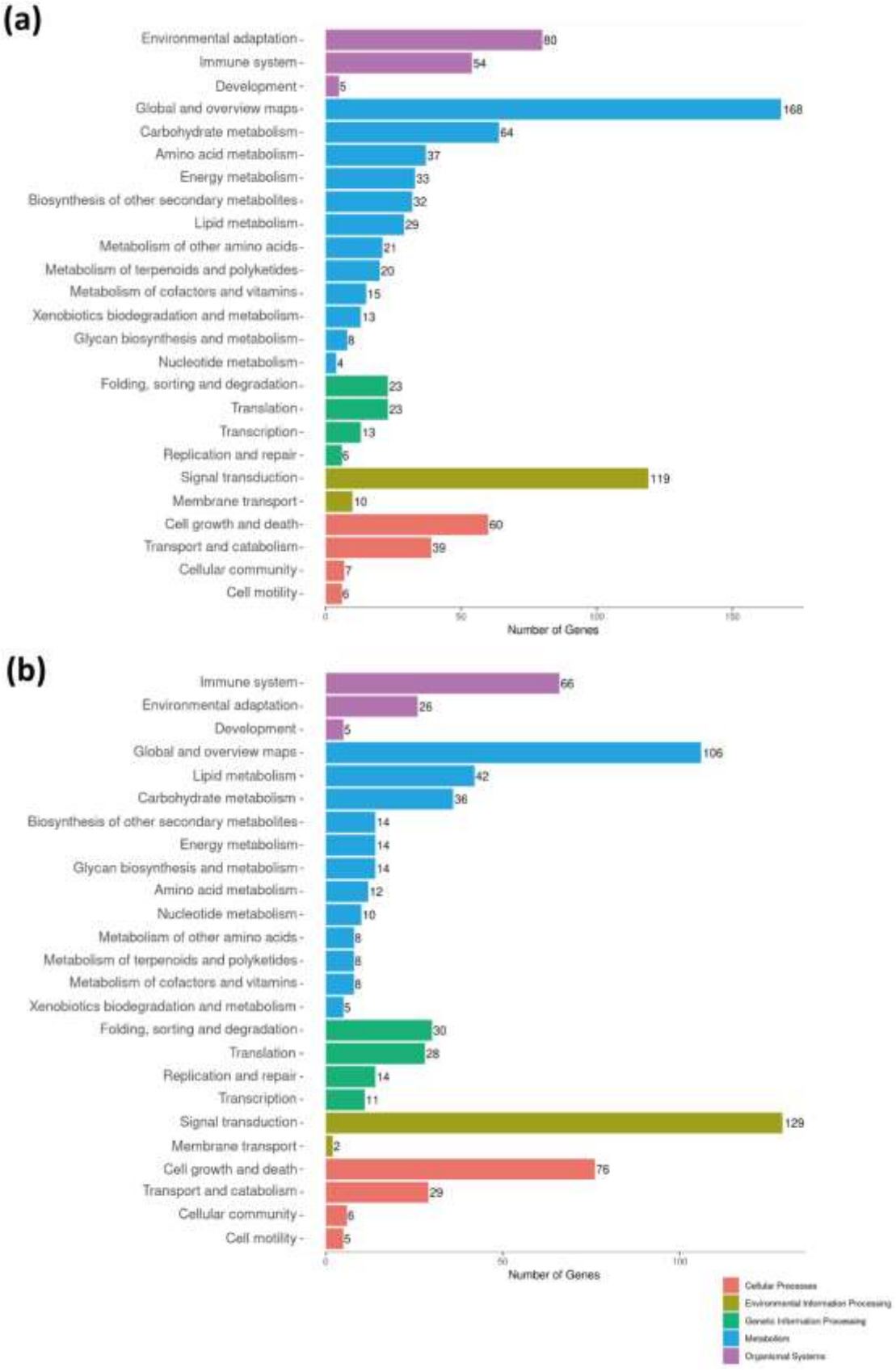

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Figure 4. Self-Organizing Maps (SOM) cluster analysis of DEGs in 12 different patterns

Clusters were obtained by the *k*-means method using the gene expression profiles of the 2,463 DEGs. The y-axis on the left side indicates the absolute value of log2(FC). KOG analysis was applied to each cluster. The x-axis on the right side represents the percentage of genes in a category.

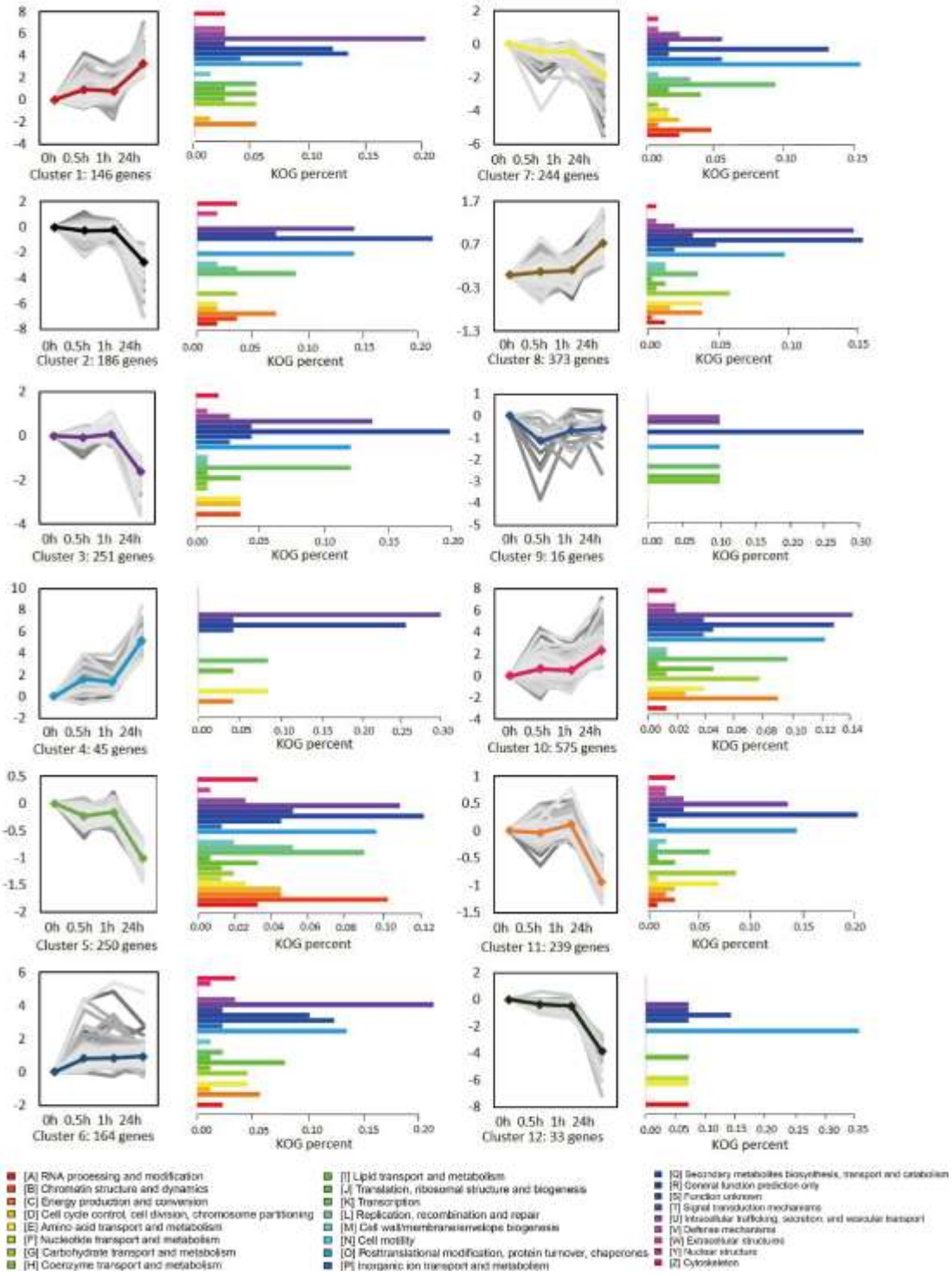

Figure 5.Classification of cold regulated transcription factors

(a) The pie chart presents 222 transcription factors sorted into 24 different families; (b) The distribution of transcription factors in up- and down- regulated categories; (c) The heat map representing 222 differentially expressed transcription factors. Columns and rows in the heat map represent samples and genes, respectively. Sample names are displayed below the heat maps. The color bar is the scale for the expression levels of each gene.

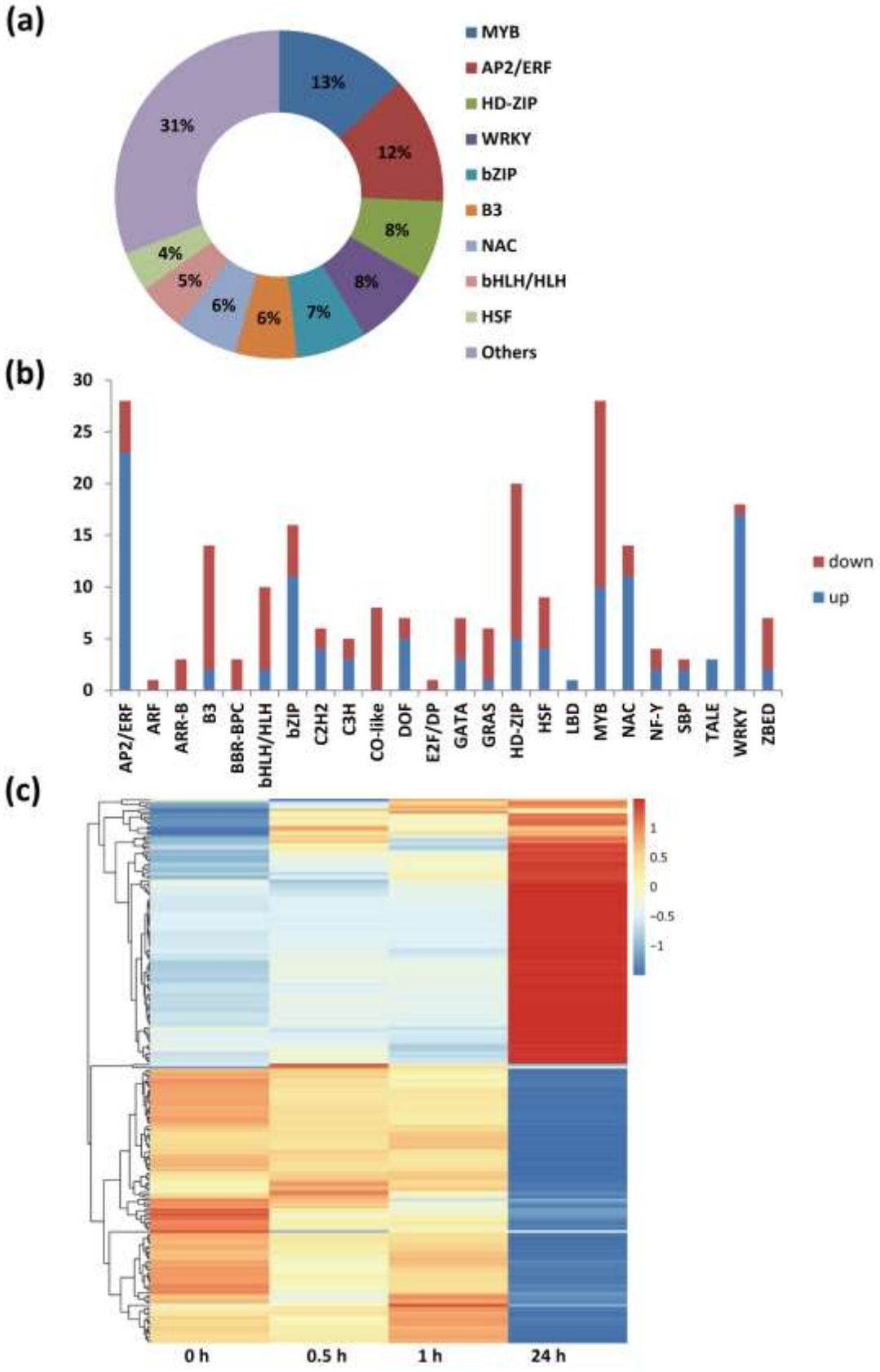

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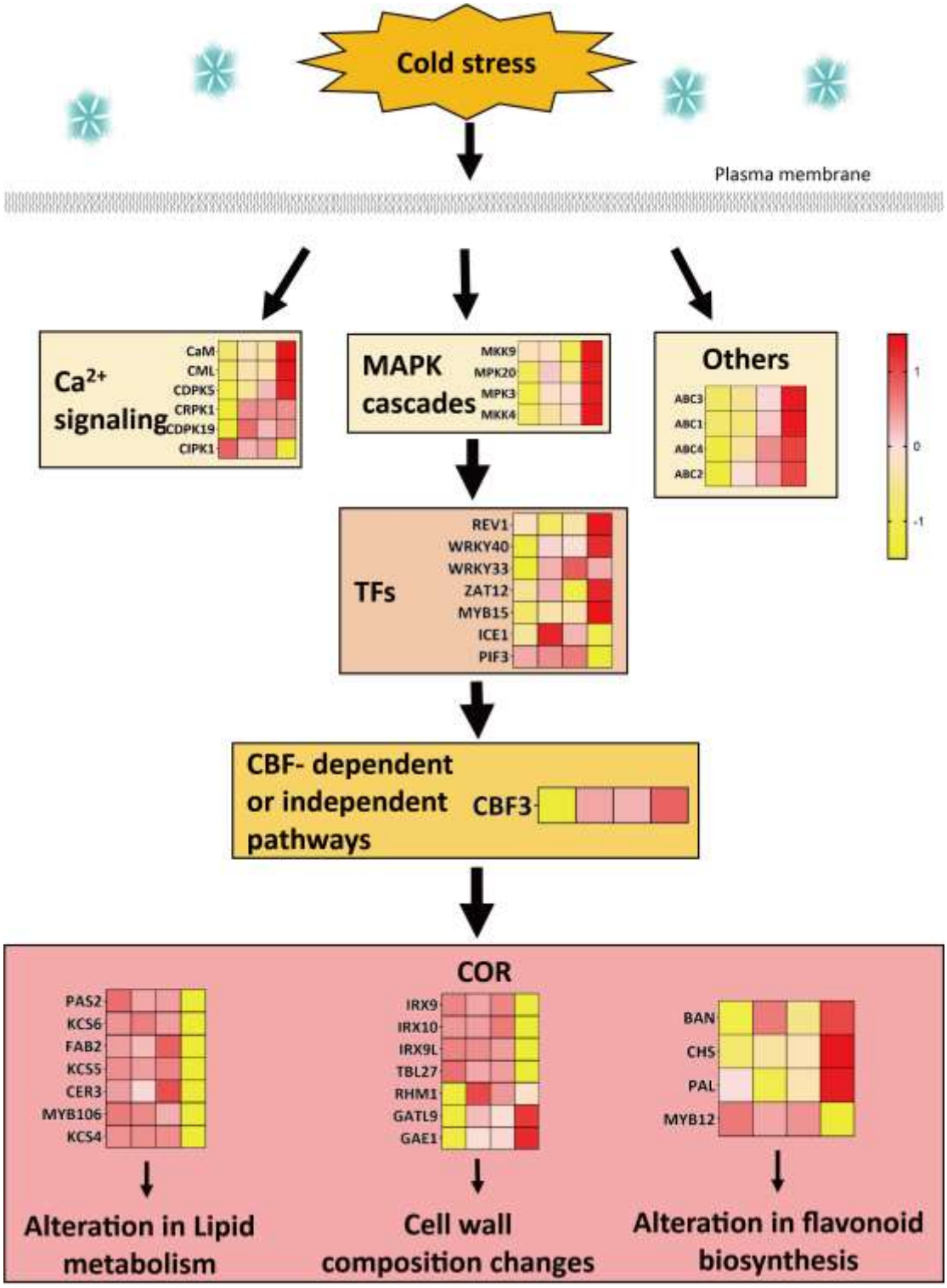
Figure 6. A model of the cold response mechanism in Moso bamboo

Cold stress is rapidly recognized through calcium signaling pathway, MAPK cascades and other pathways such as ABA signaling cascades. The signaling pathways stimulate a transcriptional cascade triggered by the CBF-dependent or CBF-independent pathways. The late responsive genes are associated with a host of metabolic activities, including antioxidants production, cell wall composition adjustments and alternations in lipid metabolism. Genes were labeled using individual heatmaps. The color bar is the scale for the expression levels of each gene on the basis of FPKM value.



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